Topically Applied *AaeIAP1* Double-Stranded RNA Kills Female Adults of *Aedes aegypti*

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ABSTRACT *Aedes aegypti* (L.) (Diptera: Culicidae) is the primary vector of both dengue and yellow fever. Use of insecticides is one of the primary ways to control this medically important insect pest. However, few new insecticides have been developed for mosquito control in recent years. As a part of our effort to develop new insecticides to control mosquitoes, an inhibitor of apoptosis protein 1 gene in *Aedes aegypti* (*AaeIAP1*) was targeted for the development of molecular pesticides. Herein, for the first time, we report that topically applied *AaeIAP1* double-stranded RNA products are able to kill female adults of *Ae. aegypti*. Our results indicate that critical pathways or genes could be targeted to develop molecular pesticides for the control of medically important diseases vectors.

KEY WORDS *Aedes aegypti*, RNA interference, double-stranded RNA, inhibitor of apoptosis, pesticide

Aedes aegypti (L.) (Diptera: Culicidae) transmits viral pathogens of humans, including yellow fever (Gillett and Ross 1955, Philip 1962, Soper 1967, Aitken et al. 1977) and dengue (Mattingly 1967, Rudnick 1967, Coleman and McLean 1973, Degallier et al. 1988), both of which can cause severe human morbidity and mortality. Although there is a safe and effective vaccine for the yellow fever virus, epidemic transmission still occurs in Africa with sporadic cases in South America (Vasconcelos et al. 2001; de Filippis et al. 2002; Valero 2003; Onyango et al. 2004a, 2004b). Dengue is the most important arboviral disease in the world and can cause an undifferentiated fever, dengue fever, dengue hemorrhagic fever, or dengue shock syndrome (Malavige et al. 2004). Annually, dengue epidemics account for several million cases and thousands of deaths worldwide (Teixeira Mda et al. 2005).

Mosquito control in many countries relies primarily on insecticides. After the introduction of synthetic organic insecticides in the 1940s and 1950s, *Ae. aegypti* was eradicated from many areas of the world. The Pan American Health Organization initiated a campaign to use DDT to eradicate *Ae. aegypti* in the Western Hemisphere in the late 1940s (Pinto Severo 1955, Fouque and Carinci 1996). By 1972, *Ae. aegypti* had been eradicated from 73% of the land area and 19 countries (Gubler 1989). However, insecticide resistance developed (Brown and Pal 1971) and the campaign ended in 1972 before the eradication goal was achieved. Furthermore, insects have developed resistance to insecticides, resulting in significant loss of efficacy to commonly used insecticides. Therefore, there is urgent need for the development of alternative insecticides to control these important disease vectors.

Apoptosis is an evolutionarily conserved pathway of cell suicide that is critical for normal development and homeostasis (Vaux et al. 1994). The key regulators of apoptosis are inhibitor of apoptosis proteins (IAPs). IAPs were originally discovered in insect baculoviruses (*Cydia pomonella* granulosis virus and *Orgyia pseudotsugata* nuclear polyhedrosis virus) (Crook et al. 1993, Birnbaum et al. 1994, Clem and Miller 1994). Since their first reports in baculoviruses, IAPs have been identified in many other organisms, such as mosquito iridescent viruses (Delhon et al. 2006), insects (Hay et al. 1995, Muro et al. 2002), yeast (Walter et al. 2006), and human (Liston et al. 1996, Ambrosini et al. 1997, Vitte-Mony et al. 1997). Many IAPs are capable of blocking apoptosis when they are overexpressed in cells of other species (Beidler et al. 1995; Hawkins et al. 1996, 1998). However, knockdown expression of IAPs through RNA interference is capable of inducing apoptosis (Li et al. 2005, Gu et al. 2006).

The discovery of RNA interference (RNAi) in eukaryotic cells is the major recent breakthrough in molecular and cell biology. The potential of RNAi to silence any gene of interest in a highly specific and efficient manner via double-stranded RNA (dsRNA) has literally revolutionized modern genetics. RNAi-based functional genomics now permits, for the first

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Table 1. Primers used for the synthesis of dsRNA products and real-time PCR

Primer name	Sequence
T7-IAP1-185F*	TAATACGACTCACTATAGGGGGCTGGAGTTATGATGGCTC
T7-IAP1-395R*	TAATACGACTCACTATAGGGTGGCCCCACGTAGTAG
T7-IAP1-375F*	TAATACGACTCACTATAGGGGTTTCTACTACGTGGGGCCA
T7-IAP1-931R*	TAATACGACTCACTATAGGGCAGCTTCCCAGTCTTTGAGG
T7-IAP1-911F*	TAATACGACTCACTATAGGGCCTCAAAGACTGGGAAGCTG
T7-IAP1-1347R*	TAATACGACTCACTATAGGGGACACAACGGACACTTGGTG
T7-Cun85-2020F*	TAATACGACTCACTATAGGGGAAATTCGCAAGCTTTAC
T7-Cun85-2640R*	TAATACGACTCACTATAGGGTGAGCGCAGATTGTGGAAG

* All primers have a 5′ T7 promoter sequence (5′-TAATACGACTCACTATAGGG-3′)

time, to evaluate the cellular role of individual gene products on a genome-wide scale in higher organisms such as mammals, presenting an alternative to the generation of animal knockouts often doomed to failure because of a lethal phenotype. RNAi has had an enormous impact on the development of novel disease models in animals, and it is likely that small interfering RNAs (siRNAs), which are the trigger molecules for RNA silencing, will become an invaluable tool for the treatment of cancer and genetic diseases (Rye and Stigbrand 2004, Grunweller and Hartmann 2005). When small double-stranded RNAs are introduced into cells, they bind to the endogenous RNAi machinery to disrupt the expression of mRNAs containing complementary sequences with high specificity. Any disease-causing gene and any cell type or tissue can potentially be targeted (Dykxhoorn and Lieberman 2006). The need for alternative insecticides and the unique mechanisms of RNA interference and apoptosis pathway triggered us to explore the potential of using RNA interference to develop molecular pesticides using RNAi technology.

To develop new effective molecular insecticides for mosquito control, we recently cloned *AaeIAP1* from the Orlando strain of *Ae. aegypti* (GenBank accession no. DQ993355) as a gene target for developing molecular pesticides. In this work, we evaluated the toxicities of three dsRNA-*AaeIAP1* products against female adults of *Ae. aegypti* through topical application method. Herein, for the first time, we report that dsRNA products of *AaeIAP1* are able to kill female adults of *Ae. aegypti.* Our results suggest that critical pathways or genes could be targeted to develop molecular pesticides for the control of medically important diseases vectors.

Materials and Methods

Ae. aegypti. The Orlando strain of *Ae. aegypti* was reared in the insectary of the Mosquito and Fly Research Unit at Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), USDA–ARS, Gainesville, FL. The Orlando strain of *Ae. aegypti* has been established in CMAVE since 1952. Female adults were used for all experiments because only females take bloodmeals and are concern of the general public. Eggs were hatched by placing a square of a paper towel with eggs in a flask filled with 1000 ml of distilled water containing 40 mg of larval diet (3:2 brewer's yeast:liver powder [MP Biomedicals, Irvine, CA]). The hatched larvae were held overnight in the flask and 200 larvae were transferred to a 4-liter plastic tray containing 2 liters of distilled water. Larval diet was added to each tray according to the following schedule: day 1, 80 mg; day 3, 40 mg; day 4, 80 mg; day 5, 120 mg; and day 6, 150 mg. Mosquitoes were reared in an environmental chamber set with a temperature profile representing a simulated summer day regime (ranging from 22 to 30°C) and 80% RH. Incandescent lighting was set to a crepuscular profile with a photoperiod of 14:10 (L:D) h, including 2 h of simulated dawn and 2 h of simulated dusk. Adults were held in a screened cage and provided 10% sucrose ad libitum. Bovine blood in 1% heparin that had been placed in a pig intestine and warmed to 37°C was provided to adults twice a week. Eggs were collected on paper towels (Vasco Brands, Elmira, NY) that lined the rim of water containers. These egg-laden papers were air dried at 27°C and 80% RH for 24 h and stored in containers with 100% humidity for 3–30 d. When needed, eggs were hatched under vacuum and larvae were reared in containers as described above.

***Ae. aegypti* RNA Extraction and cDNA Synthesis.** The adults of Orlando strain of *Ae. aegypti* were used for total RNA extraction. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Total RNA was resuspended in diethyl pyrocarbonate-treated water and stored at −80°C. A 1.0% denaturing agarose/formaldehyde gel was used to test the integrity and quantity of the RNA. Poly(A)$^+$ RNA was isolated using Oligotex-dT suspension (QIAGEN, Valencia, CA). Cloning the full-length *AaeIAP1* gene was carried out using Generacer kit (Invitrogen) as described by the manufacturer. The first strand cDNAs were synthesized with avian myeloblastosis virus reverse transcriptase (Invitrogen) by using *Ae. aegypti* mRNAs as templates.

dsRNA Synthesis. Four double-stranded RNA products were made using the MEGAscript RNAi kit (Ambion, Austin, TX). The first dsRNA product (dsRNA-IAP1-B) was designed to cover the N-terminal portion of *AaeIAP1* and part of its first baculovirus IAP repeat (BIR) domain. It was made by amplifying plasmid DNA containing a cloned 252-bp fragment with the T7-IAP-183 F/T7-IAP-395R primers (Table 1) that had T7 promoter sequence (5′-TAATACGACTCACTATAGGG-3′) added to the 5′ end of each primer. The resulting template was then transcribed for 16 h

by using T7 RNA polymerase following the manufacturer's protocol (Ambion). The second dsRNA product (dsRNA-IAP1-C) was designed to produce the middle portion of *AaeIAP1* that contains the second BIR domain and the rest of the first BIR domain. It was made by amplifying plasmid DNA containing a cloned ≈556-bp fragment *AaeIAP1* with the T7-IAP-375 F/T7-IAP-931R primer set (Table 1). The resulting template was then transcribed for 16 h. The third dsRNA product (dsRNA-IAP1-D) contained the rest of the *AaeIAP1* that contains a C-terminal RING domain. Plasmid DNA containing a cloned ≈436-bp fragment of *AaeIAP1* was amplified using T7-IAP-911 F/T7-IAP-1347R primer set (Table 1). The resulting template was then transcribed for 16 h. All primers for the synthesis of dsRNA products of *AaeIAP1* were designed based on the sequence of the inhibitor of apoptosis-1 like protein mRNA (GenBank accession no. AY559037). The fourth dsRNA was designed to produce a nontargeting dsRNA control using Cun85 from *Culex nigripalpus* baculovirus (CuniNPV) as template. Primer set T7-Cun85–2020 F/T7-Cun85–2640R (Table 1) was designed based on the sequence of the Cun85 gene published in the genome of CuniNPV (GenBank accession no. AF403738). The resulting template was then transcribed for 16 h. DNA and ssRNA were removed and the dsRNA products were then purified following the manufacturer's protocol (Ambion). All plasmid DNA used as templates for dsRNA synthesis were sequenced by automated sequencing (DNA Sequencing Facility, University of Florida, Gainesville, FL).

Adult Bioassays. To determine the toxicity of each dsRNA-IAP1 product against female adults of *Ae. aegypti*, each dsRNA product was diluted in acetone and topically applied to individual mosquitoes. Before insecticide application, 5–7 d-old females were briefly anesthetized for 30 s with carbon dioxide and placed on a 4°C chill table (BioQuip Products, Rancho Dominguez, CA). A droplet of 0.5 μl of dsRNA molecular insecticide solution was applied to the dorsal thorax by using a 700 series syringe and a PB 600 repeating dispenser (Hamilton, Reno, NV). After treatment, mosquitoes were kept in plastic cups and supplied with 10% sucrose solution for 24 h before mortality was recorded. Temperature and humidity were maintained at 26°C and 80% RH, respectively. Each bioassay was conducted at 27°C and 80% RH and replicated three times. One way analysis of variance (ANOVA) using Holm–Sidak method (a more powerful method than the Turkey and Bonferroni tests, recommended as the first line procedure for most multiple comparison testing), and SigmaStat software (Systat Software, Inc., Point Richmond, CA) was used to compare mortalities among different treatments with a criterion of $P \leq 0.001$.

Real-Time Quantitative Polymerase Chain Reaction (PCR). Twenty-four hours after topical application, female *Ae. aegypti* were froze in −80°C for subsequent RNA extraction. Total RNA was prepared and reverse transcribed as described above. Quantitative PCR was performed with SYBR Green PCR Master Mix on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative level of *AaeIAP1* mRNA expression was determined by dividing the threshold cycle (C_t) of each sample by that of the *Ae. aegypti* actin gene (GenBank accession no. DQ440059), the calibrator or internal control, as per the formula $\Delta C_t = C_t$ (sample) $- C_t$ (calibrator). Primers for the amplification of the *AaeIAP1* gene were the forward primer IAP-911 F (5′-CCTCAAAGACTGGGAAGCTG-3′) and the reverse primer IAP-1133R (5′-TGACTGAAGCGAGGATGTTG-3′). Primers for the amplification of the actin gene were forward primer set Actin-152 F (5′-AGGACTCGTACGTCGGTGAC-3′) and Actin-590R (5′-CGTTCAGTCAGGATCTTC-3′). The expression levels of *AaeIAP1* mRNA compared with actin mRNA level was calculated as ΔC_t, where $\Delta C_t = C_t$ (*AaeIAP1*) $- C_t$ (actin). The comparative expression levels between samples treated with or without dsRNA-IAP1 products were then calculated by the formula $2^{\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t$ (sample treated with dsRNA) $- \Delta C_t$ (samples without dsRNA treatment).

DNA Fragmentation Assay. Thirty adult female mosquitoes were treated with or without dsRNA products by using acetone as carrier and maintained at 26°C and 80% RH, respectively. Twenty-four hours posttreatment, mosquitoes were placed on dry ice and then harvested to assay DNA fragmentation, one of the hallmarks of cells undergoing apoptosis. The DNA fragmentation assay was performed with a well-established method with slight modification (Fombonne et al. 2006). Briefly, mosquitoes treated with or without double-stranded RNA were three times with Dulbecco's phosphate-buffered saline. The mosquitoes were then homogenized in binding and lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, and 20% Triton X-100, pH 4.4) and incubated for 10 min at 25°C. Isopropanol was then added to the lysate. The mixture of lysate and isopropanol was then subjected to centrifugation. Supernatant was then passed through a filter tube provided by the Apoptotic DNA ladder kit (Roche Applied Science, Mannheim, Germany), and the nucleic acid purified by the kit was then analyzed by gel electrophoresis on a 1% agarose gel in Tris-acetate buffer (40 mM Tris acetate and 1 mM EDTA). After electrophoresis, DNA was visualized by staining with ethidium bromide. Gels were photographed with a digital camera (Olympus, Melville, NY).

Results and Discussion

To prepare template for dsRNA synthesis, *AaeIAP1* gene was cloned from the Orlando strain of *Ae. aegypti* and the sequence was deposited to GenBank (accession no. DQ993355). The quality of the nonsilencing dsRNA control Cun85 and the three *AaeIAP1* dsRNA products (dsRNA-IAP1-B, dsRNA-IAP1-C, and dsRNA-IAP1-D) was analyzed by 1% agarose gel electrophoresis, along with their DNA templates (Fig. 1). The expected size for the nonsilencing dsRNA control Cun85 was 621 bp. The expected sizes for the three silencing *AaeIAP1* dsRNA products (dsRNA-

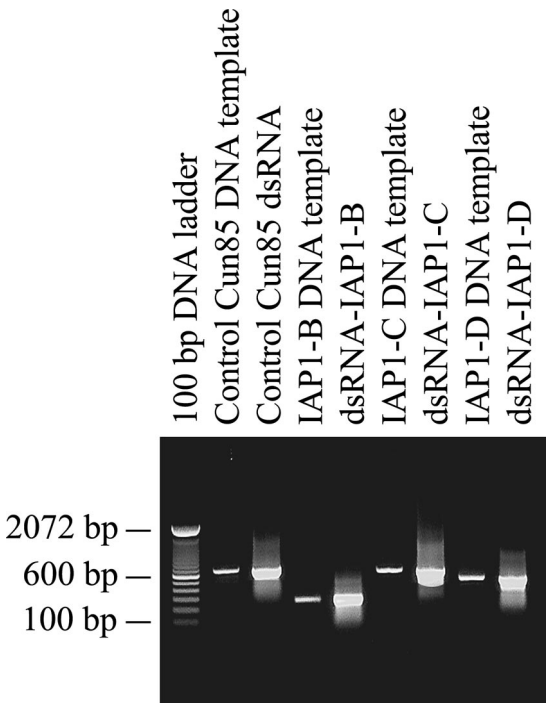

Fig. 1. Agarose gel (1%) analysis of dsRNA synthesized. The amount of the 100-bp DNA marker was 0.5 μg. The amount of the DNA templates loaded in the gel was 0.5 μg. The amount of the control dsRNA product Cun85 was 2.35 μg. The amount of the *AaeIAP1* dsRNA products loaded in the gels was 2.02, 2.26, and 2.02 μg, respectively.

IAP1-B, dsRNA-IAP1-C, and dsRNA-IAP1-D) were 211, 557, and 437 bp, respectively. As shown in Fig. 1, all four dsRNA products showed up sharp bands, indicating that dsRNA products were synthesized successfully. The concentrations for the four dsRNA products were 0.784, 0.672, 0.752, and 0.674 μg/μl, respectively. Figure 1 also showed that the dsRNA products migrated at similar speed as their DNA templates.

To determine whether dsRNA-*AaeIAP1* products were able to kill female adults of *Ae. aegypti*, topical application bioassays were performed. Using acetone as a carrier for the delivery of dsRNA, at 3 h posttopical application, dsRNA-IAP1-B, dsRNA-IAP1-C, and dsRNA-IAP1-D caused 8.67 ± 2.08, 5.67 ± 2.31, and 8.00 ± 1.73% mortality, respectively (Table 2), with no significant difference among the three treatments. However, the combination of the three dsRNA products of *AaeIAP1* caused significantly higher mortality (14.00 ± 3.00%) at 3 h postapplication. In contrast, the acetone control and the application of nontargeting dsRNA-Cun85 did not cause any mortality (Table 2). At 12 h posttopical application, the mortality caused by the combination of the three dsRNA products of *AaeIAP1* was increased to 30 ± 4.36%, whereas the mortality caused by the individual dsRNA products (dsRNA-IAP1-B, dsRNA-IAP1-C, and dsRNA-IAP1-D) was 11.00 ± 1.00, 9.00 ± 1.73%, and 15.33 ± 4.04%, respectively (Table 2). At 24-h post topical application, the mortality caused by the combination of the three dsRNA products of *AaeIAP1* was increased to 42.00 ± 4.58%, whereas the mortality caused by the individual dsRNA products (dsRNA-IAP1-B, dsRNA-IAP1-C, and dsRNA-IAP1-D) was 15.67 ± 1.53%, 14.33 ± 2.31%, and 19.00 ± 3.46%, respectively (Table 2). At all time points, the combination of three dsRNA-IAP1 products caused significantly higher mortality.

To understand whether different carrier for the dsRNA delivery will have an effect on bioassay results, TransIT-siQUEST transfection reagent (Mirus Bio Corporation, Madison, WI) was used as another carrier to study the toxicities of dsRNA-IAP1 products. TransIT-siQUEST reagent has been successfully used to transfect cell cultures with siRNA (Grayson et al. 2006). It is a proprietary cationic polymer/lipid formulation supplied in ethanol. Using TransIT-siQUEST transfection reagent as a carrier for the delivery of dsRNA, at 3 h posttopical application, dsRNA-IAP1-D caused significantly higher mortality (28.00 ± 4.58%) than the other three *AaeIAP1* dsRNA treatments (dsRNA-IAP1-B, dsRNA-IAP1-C, and dsRNA-IAP1-B+C+D) (Table 3). At 12 h posttopical application, the mortality caused by dsRNA-IAP1-D was increased to 32.67 ± 4.04% (Table 3). Mortalities caused by the other three AaeIAP1 dsRNA treatments (dsRNA-IAP1-B, dsRNA-IAP1-C, and dsRNA-IAP1-B+C+D) also were increased (Table 3). At 24 h posttopical application, the highest mortality caused by dsRNA-IAP1-D was increased to 48.33 ± 4.04%, followed by

Table 2. Toxicities of dsRNA-IAP1 products against female adults of *Ae. aegytpi* by using acetone as carrier

Solution	Ratio of acetone:dsRNA	Mean mortality ± SD (%) [dead/total]		
		3 h	12 h	24 h
Acetone		0 [0/30; 0/30; 0/30]	0 [0/30; 0/30; 0/30]	0 [0/30; 0/30; 0/30]
Acetone + dsRNA-Cun85	3:1	0 [0/30; 0/30; 0/30]	0 [0/30; 0/30; 0/30]	0 [0/30; 0/30; 0/30]
Acetone + dsRNA-IAP1-B	3:1	8.67 ± 2.08 [2/30; 2/25; 3/28]	11.00 ± 1.00 [3/30; 3/25; 3/28]	15.67 ± 1.53 [5/30; 4/25; 4/28]
Acetone + dsRNA-IAP1-C	3:1	5.67 ± 2.31 [2/30; 1/30; 2/30]	9.00 ± 1.73 [2/30; 3/30; 3/30]	14.33 ± 2.31 [4/30; 4/30; 5/30]
Acetone + dsRNA-IAP1-D	3:1	8.00 ± 1.73 [2/30; 3/30; 2/30]	15.33 ± 4.04 [4/30; 4/30; 6/30]	19.00 ± 3.46 [5/30; 5/30; 7/30]
Acetone + dsRNA-IAP1-B+C+D	3:1:1:1	14.00 ± 3.00 [3/28; 3/22; 5/30]	30.00 ± 4.36 [7/28; 7/22; 10/30]	42.00 ± 4.58 [12/28; 10/22; 11/30]

Table 3. Toxicities of dsRNA-IAP1 products against female adults of *Ae. aegytpi* by using siQUEST as carrier

Solution	Ratio of siQUEST:dsRNA	Mean mortality ± SD (%) [dead/total]		
		3 h	12 h	24 h
siQUEST		0 [0/29; 0/28; 0/30]	0 [0/29; 0/28; 0/30]	0 [0/29; 0/28; 0/30]
siQUEST + dsRNA-Cun85	3:1	0 [0/23; 0/27; 0/30]	0 [0/23; 0/27; 0/30]	0 [0/23; 0/27; 0/30]
siQUEST + dsRNA-IAP1-B	3:1	8.00 ± 1.00 [2/25; 2/30; 2/23]	14.00 ± 2.65 [3/25; 4/30; 4/23]	31.67 ± 5.51 [8/25; 11/30; 6/23]
siQUEST + dsRNA-IAP1-C	3:1	6.83 ± 0.27 [2/28; 2/30; 2/30]	11.33 ± 1.53 [3/28; 3/30; 4/30]	16.00 ± 4.58 [3/28; 5/30; 6/30]
siQUEST + dsRNA-IAP1-D	3:1	28.00 ± 4.58 [7/30; 8/28; 9/28]	32.67 ± 4.04 [11/30; 9/28; 8/28]	48.33 ± 4.04 [16/30; 13/28; 13/28]
siQUEST + dsRNA-IAP1-B+C+D	3:1:1:1	5.00 ± 2.65 [1/30; 1/28; 2/25]	17.00 ± 3.00 [5/30; 4/28; 5/25]	32.67 ± 7.51 [10/30; 7/28; 10/25]

the treatment with the combination of the three dsRNA-IAP1 products (32.67 ± 7.51%) and dsRNA-IAP1-B (31.67 ± 5.51%). The dsRNA-IAP1-C caused significantly lower mortality (16.00 ± 4.58%) at 24-h time point (Table 3). In contrast, the acetone control and the application of nontargeting dsRNA-Cun85 did not cause any mortality at all time points (Table 3), suggesting that the mortality was caused by AaeIAP1 dsRNA through specific RNA interference.

Because various *AaeIAP1* dsRNA products caused different mortalities to female *Ae. aegypti*, we investigated the gene silencing effects caused by the different treatments of *AaeIAP1* dsRNA products through real-time quantitative PCR by using actin as an internal control. As shown in Table 4, when acetone was used as the carrier for dsRNA delivery, treatment of *Ae. aegypti* with the combination of the three *AaeIAP1* dsRNA products suppressed the expression of *AaeIAP1* at the highest level (6.6-fold) compared with other three AaeIAP1 dsRNA treatments. In contrast, little or no significant *AaeIAP1* gene suppression was observed in mosquitoes treated with or without nonsilencing Cun85 dsRNA product (Table 4). This result is consistent with the mortality results in Table 2. Similarly, when siQUEST was used as the carrier for dsRNA delivery, *AaeIAP1* mRNA level was suppressed at the highest level (8.07-fold) compared with the other three dsRNA-IAP1 treatments (Table 4), which is consistent with the mortality results in Table 3, where the highest mortality was caused by the treatment of dsRNA-IAP1-D. In contrast, *AaeIAP1* transcript levels were not significantly reduced in mosquitoes treated with nontargeting Cun85-A dsRNA, further suggesting that the mortality was specifically caused by *AaeIAP1* dsRNA interference.

To understand whether the application of dsRNA-IAP1 products actually induced apoptosis, thus caused mortality, DNA fragmentation assays were performed. The effect of *AaeIAP1* dsRNA products on the induction of apoptosis were shown in Fig. 2. The DNA fragmentation was obvious in mosquitoes treated with *AaeIAP1* dsRNA. In contrast, DNA fragmentation effects were not observed in either the mosquitoes treated with a nonsilencing dsRNA product Cun85 (from CuniNPV virus) or without any dsRNA products, suggesting that the mortality caused by dsRNA-IAP1 treatments were caused by apoptosis through specific RNA interference.

The discovery of RNAi in eukaryotic cells is the major recent breakthrough in molecular and cell biology. The potential of RNAi to silence any gene of interest in a highly specific and efficient manner via dsRNA has literally revolutionized modern genetics. However, introducing siRNAs into cells and organisms remains an important obstacle. In insects, four methods (injecting, feeding, soaking, and in vivo delivery

Table 4. *AaeIAP1* mRNA expression levels in female *Ae. aegytpi* exposed to dsRNA-IAP1 products by using acetone or TransIT-siQUEST transfection reagent as carrier

Solution	Carrier:dsRNA	Amount of dsRNA (ng per female)	Decreased *IAP1* level (-fold ± SD)[a]
Acetone		0	
Acetone + dsRNA-Cun85	3:1	98	1.01 (0.08)
Acetone + dsRNA-IAP1-B	3:1	84	1.92 (0.43)
Acetone + dsRNA-IAP1-C	3:1	94	2.00 (0.60)
Acetone + dsRNA-IAP1-D	3:1	84	2.41 (0.30)
Acetone + dsRNA-IAP1-B+C+D	3:1:1:1	B:56/C:63/D:56	6.60 (0.24)
siQUEST		0	
siQUEST + dsRNA-Cun85	3:1	98	0.80 (0.01)
siQUEST + dsRNA-IAP1-B	3:1	84	2.76 (0.72)
siQUEST + dsRNA-IAP1-C	3:1	94	1.58 (0.25)
siQUEST + dsRNA-IAP1-D	3:1	84	8.07 (0.64)
siQUEST + dsRNA-IAP1-B+C+D	3:1:1:1	B:56/C:63/D:56	3.10 (1.61)

[a] Decreased *AaeIAP1* levels were detected by quantitative PCR by using actin as an internal control and compared with that in acetone or siQUEST-treated control samples.

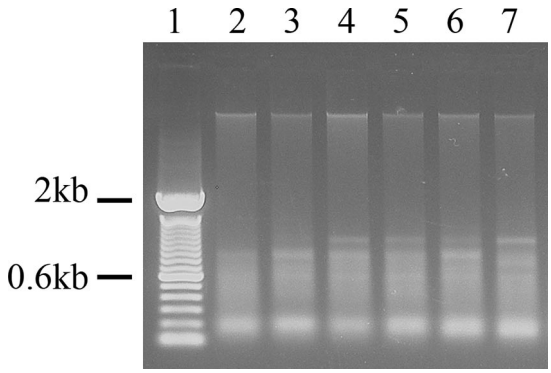

Fig. 2. DNA fragmentation assay analyzed by 1% agarose gel electrophoresis. Lane 1, 100-bp DNA marker; lane 2, acetone-treated control; lane 3, acetone + dsRNA-Cun85 treated; lane 4, acetone + dsRNA-IAP1-B treated; lane 5, acetone + dsRNA-IAP1-C treated; lane 6, acetone + dsRNA-IAP1-D treated; and lane 7, acetone + dsRNA-IAP1-B+C+D treated.

using virus) have been used successfully to knockdown specific gene expression by RNAi (Araujo et al. 2006). Topical application using dsRNA directly against an insect has never been reported. Herein, for the first time, we report that topically applied dsRNA-AaeIAP1 products are able to induce mortality against female adults of *Ae. aegypti.* Because dsRNA-IAP1 could cause 100% mortality to female adults of *Ae. aegypti* through microinjection (our unpublished data), we conclude that specific formulation could be developed to efficiently carry dsRNA into insects and dsRNA products could be sprayed as conventional chemical pesticides. To generate enough dsRNA at levels required for vector control in the real world, we are currently exploring the possibility to generate dsRNA in mutant bacteria strain such as HT115 (Timmons et al. 2001).

Because apoptosis is an evolutionary conserved pathway and inhibitors of apoptosis protein are also conserved among different species, one might be concerned that one insecticide targeting this evolutionary conserved pathway might be very nonspecific and affect nontargets. However, dsRNA-based molecular pesticides are targeting the RNAs in one species, not the proteins in that species. Therefore, a specific segment of RNA that is not shared among species will be targeted to achieve the RNA interference effect.

Using siQUEST as a carrier to topically deliver the three dsRNA products of AaeIAP1, the highest mortality (48.33 ± 4.04%) was caused by dsRNA-IAP1-D, suggesting that the 3′-end dsRNA product of *AaeIAP1* might have the highest efficiency in knocking down AaeIAP1 protein level. This result is not surprising in that *AaeIAP1* could have alternative splicing isoforms. Alternative splicing isoforms of IAP1 have been confirmed in murine (Mosley and Keri 2006), in which the murine c-IAP1 isoforms are able to enhance the ability to inhibit apoptosis. For both dsRNA-IAP1-B and dsRNA-IAP1-D, using siQUEST as carrier achieved higher mortalities than using acetone as carrier, suggesting that siQUEST was able to deliver these two dsRNA products better than acetone. Currently, we are working on producing more dsRNA products targeting different critical pathways and using different formulation to achieve the best toxicities of dsRNA molecular pesticides.

In conclusion, we evaluated the insecticidal activities of three dsRNA products of *AaeIAP1* as molecular pesticides against female *Ae. aegypti* through topical application. This is the first report that dsRNA could be topically applied to adult mosquito as molecular pesticides. These preliminary results will be useful in guiding further improvement in the development of new molecular insecticides for the control of medically important disease vectors.

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